Remarks

Claims 1-6, 9-25 and 32-42 are pending. Claims 3, 9-10, 17, 24, 25 and 40-42 are stated to be under consideration in the Office Action. Claim 3 is amended to make it independent with support in claim 10 as filed. Claim 17 is amended to make it independent with support in claim 14 as filed. Claim 40 is amended to delete the phrase "and wherein the mosaic polypeptide is not the HCV polyprotein" with support as indicated in the amendment submitting claim 40. New claim 43 depends from claim 3 and includes the negative limitation. New claim 44 depends from claim 40 and includes the negative limitation. Support for the negative limitation is provided below. These amendments add no new matter and their entry is respectfully requested.

Regarding claim 23, it was not intended to be cancelled. Rather it should have been indicated to be withdrawn as is indicated in the current listing of claims.

The present Office Action states that any ground for rejection that is not repeated has been removed. Thus, applicants understand that all of the former rejections under 35 U.S.C. § 102, that is, over Jin et al. (Arch. Biochem. Biophy. 1995, Vol. 323, No. 1, pp. 47-53), Yagi et al. (Biol. Pharm. Bull. 1996, Vol. 19, No. 10, pp. 1254-1260), Houghton et al. (US Patent NO. 5,683,846A), Barrera et al. (Vox Sang 1995, Vol. 68, pp. 15-18) and Okayma et al. (EP 464 287A1), are withdrawn.

In the previous Office Action, claims 3, 16, 17 and 40 were stated to be free of the art, given the failure of the prior art to teach or reasonably suggest a mosaic polypeptide particularly comprising amino acid residues 1471-1573 of HCV NS3 of SEQ ID NO:2 or amino acid 1-120 of SEQ ID NO:1. Claims 3 and 17 were indicated to be rejected because they depend from rejected base claims 10 and 14, respectively. Thus, with the present amendment of claims 3 and 17 to make them independent, these claims are in condition for allowance. Claim 40 was indicated to be rejected only on the basis of the new matter rejection. Thus, with the present amendment to claim 40 to delete the supposed new matter, this claim is allowable.

Furthermore, new claims 43 and 44, retain the negative limitation of claims 3 and 40, respectively, prior to the amendments to those claims herein. Thus, to the extent that the negative limitation avoids certain art, this art is avoided for new claims 43 and 44.

New Matter Objection and Rejection

The amendment filed February 19, 2003 remains objected to under 35 U.S.C. § 132, and claims 9-10 and 40 are rejected under 35 U.S.C § 112, first paragraph as improperly reciting a negative limitation. More specifically, the Office asserts that the specification does not provide a clear explanation as to what "wherein the mosaic polypeptide is not a HCV polypeptide" means or what the skilled person would understand this concept to refer to.

Applicants' appreciate the Examiner's clarification of the Office's position on this issue. In view of the explanation of this rejection in the current Office Action, it appears that the

concern with applicants' negative limitation has to do with the content of what is being excluded, rather than the legal propriety of such exclusions. This issue of content is addressed in both the specification and in the art at the time the application was filed.

However, applicants must first point out that this rejection is unclear in the recitation of "wherein the mosaic polypeptide is not an HCV <u>polypeptide</u>." This is not what the amended claim recites. Rather the claim recites "wherein the mosaic polypeptide is not the HCV <u>polyprotein</u>." The differences in these two clauses are significant. As is demonstrated below, everyone of skill in the relevant art knows what "the HCV <u>polyprotein</u>" is, whereas "a HCV polypeptide" could be any polypeptide of HCV. Since the scope and meaning of these terms are so very different, the Office Action's statements regarding excluding an "HCV polypeptide" are not germane to the present invention.

Since applicants understand the real question to be the meaning of the term "the HCV polyprotein," this question is specifically address in this and the following paragraphs. The specification describes "the HCV polyprotein" as follows: "[t]he HCV genome consists of a 94 kb positive sense RNA molecule that contains one large open reading frame capable of encoding a polyprotein of 3010 or 3011 amino acids" (page 1, lines 17-19). The HCV polyprotein is a 3010 or 3011 amino acid protein encoded by the HCV open reading frame. Thus, the question of what is excluded from the claims is taught in unequivocal terms in the present specification.

The relevant art treats the term "HCV polyprotein" as an art-recognized concept. In fact, on PubMed alone there are 105 publications that use this term in their abstracts. There are at least 32 publications from the art at the time the present application was filed that use this term in their abstracts. This search is attached as Exhibit 1. Illustrative examples of articles available prior to the present application's filing date that refer unambiguously to the "HCV polyprotein" and are provided as Exhibits 2-13. As exemplary only, applicants also provide a full reference of Stempniak et al. (Exhibit 14) that uses the term HCV polyprotein unambiguously and with the same meaning as recited in applicants' specification. The fact that this term appears in the abstracts and backgrounds of many of these papers means that it is not an uncommon or indefinite term. The exhibits submitted herewith provide uncontroverted evidence that the meaning of "the HCV polyprotein" was well-known in this art at the time the application was filed. Thus, the question raised by the Examiner about the meaning of what is being excluded from the claims is clearly addressed in the art.

As the Office has acknowledged, the only requirement for a valid negative limitation is that what is to be excluded must have been disclosed. As noted in the Office Action, the question in this case is whether the meaning of the exclusion is known either from the specification or the art. The negative limitation excludes the HCV polyprotein by stating "wherein the mosaic polypeptide is not the HCV polyprotein." There does not appear to be any doubt about what as to what the terms "wherein" and "is not" mean.

Regarding the meaning of "mosaic polypeptide," the specification defines "mosaic polypeptides" as "artificial composite proteins constructed from diagnostically relevant antigenic regions derived from different HCV proteins" (page 2, lines 22-24 and page 4, lines 27-29). This provides a definition of "mosaic polypeptide" that is unambiguous. Accordingly, the claims are directed to an artificial composite protein constructed from diagnostically relevant antigenic regions derived from different HCV proteins (i.e., a mosaic polypeptide), wherein this artificial composite protein is not the well-recognized HCV polyprotein. Since this is definite and understandable to one of skill in the art, there is no basis to question the meaning of either what is claimed or what is excluded from the claims.

Because the application (and the art) teach what is meant by "mosaic polypeptide" and "HCV polyprotein," the recitation in the claims of a "mosaic polypeptide, wherein the mosaic polypeptide is not the HCV polyprotein," does not constitute new matter and should not have been rejected under 35 U.S.C. 112, first paragraph for the negative limitation. Thus claims 9, 10 and 40 are not properly rejected on this basis, and withdrawal of the rejection is respectfully requested.

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Respectfully submitted,

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Expression of processed core protein of hepatitis C virus in mammalian cells.
J Virol. 1991 Jun;65(6):3015-21.
PMID: 1709694 [PubMed - indexed for MEDLINE]

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Expression of processed core protein of hepatitis C virus in mammalian cells.

Harada S, Watanabe Y, Takeuchi K, Suzuki T, Katayama T, Takebe Y, Saito I, Miyamura T.

Department of Medical Entomology, National Institute of Health, Tokyo, Jap

A structural protein of hepatitis C virus (HCV) was expressed in monkey CO cells under the control of an exogenous promoter, and a protein of 22 kDa wa identified by immunoblot analysis. This protein (p22), which was produced b processing in COS cells, reacted specifically to sera of chronic hepatitis C patients, and its coding region was mapped at the most amino-terminal part o the HCV polyprotein. These results suggested that the p22 protein is the nucleocapsid (core) protein of HCV. Moreover, the assay detecting antibody p22 was found to be useful for early diagnosis of HCV infection.

PMID: 1709694 [PubMed - indexed for MEDLINE]

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Expression and identification of hepatitis C virus polyprotein cleavage products.

Grakoui A, Wychowski C, Lin C, Feinstone SM, Rice CM.

Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110-1093.

Hepatitis C virus (HCV) is the major cause of transfusion-acquired non-A, n B hepatitis. HCV is an enveloped positive-sense RNA virus which has been classified as a new genus in the flavivirus family. Like the other two genera i this family, the flaviviruses and the pestiviruses, HCV polypeptides appear t be produced by translation of a long open reading frame and subsequent proteolytic processing of this polyprotein. In this study, a cDNA clone encompassing the long open reading frame of the HCV H strain (3,011 amin acid residues) has been assembled and sequenced. This clone and various truncated derivatives were used in vaccinia virus transient-expression assays map HCV-encoded polypeptides and to study HCV polyprotein processing. HCV polyproteins and cleavage products were identified by using convalesc human sera and a panel of region-specific polyclonal rabbit antisera. Similar results were obtained for several mammalian cell lines examined, including t human HepG2 hepatoma line. The data indicate that at least nine polypeptide are produced by cleavage of the HCV H strain polyprotein. Putative structura proteins, located in the N-terminal one-fourth of the polyprotein, include the capsid protein C (21 kDa) followed by two possible virion envelope proteins E1 (31 kDa) and E2 (70 kDa), which are heavily modified by N-linked glycosylation. The remainder of the polyprotein probably encodes nonstructu proteins including NS2 (23 kDa), NS3 (70 kDa), NS4A (8 kDa), NS4B (27 kDa), NS5A (58 kDa), and NS5B (68 kDa). An 82- to 88-kDa glycoprotein which reacted with both E2 and NS2-specific HCV antisera was also identifi (called E2-NS2). Preliminary results suggest that a fraction of E1 is associate with E2 and E2-NS2 via disulfide linkages.

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Both NS3 and NS4A are required for proteolytic processing of hepatitis C virus nonstructural proteins.

Failla C, Tomei L, De Francesco R.

Istituto di Ricerche di Biologia Molecolare P. Angeletti-Pomezia, Rome, Ital

The proteolytic cleavages at the NS3-NS4A, NS4A-NS4B, NS4B-NS5A, an NS5A-NS5B junctions of hepatitis C virus (HCV) polyprotein are effected b the virus-encoded serine protease contained within NS3. Using transient expression in HeLa cells of cDNA fragments that code for regions of the HC polyprotein, we studied whether viral functions other than NS3 are required 1 proteolytic processing at these sites. We found that, in addition to NS3, a C-terminal 33-amino-acid sequence of the NS4A protein is required for cleavag at the NS3-NS4A and NS4B-NS5A sites and that it accelerates the rate of cleavage at the NS5A-NS5B junction. In addition, we show that NS4A can activate the NS3 protease when supplied in trans. Our data suggest that HCV NS4A may be the functional analog of flavivirus NS2B and pestivirus p10 proteins.

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Translation of hepatitis C virus genome.

Ali N, Wang C, Siddiqui A.

Department of Microbiology, University of Colorado Health Sciences Cente
Denver 80262, USA.

Translation of the human hepatitis C virus (HCV) RNA genome occurs by internal ribosome entry through the 5' end (5' noncoding region) in a cap-independent fashion. The relatively long stretch of this noncoding region contains multiple initiation codons that are apparently not used for translatio Translation of the HCV polyprotein is initiated instead from an AUG located nt 342. Using computer-assisted analysis (and subsequently substantiated by enzymatic probing), a complex secondary and tertiary structure of the 5' noncoding region (5'NCR) has been predicted. Based on an RNA folding mo proposed by Brown et al. (1992), a detailed mutational analysis carried out identified the key secondary structural regions that are of functional significance in translational control. Maintenance of a helical structural elem relevant to an oligopyrimidine tract is essential for internal initiation. A putat coaxial stacking or a pseudoknot structure upstream of the initiator AUG see to be central to an internal ribosome entry site (IRES)-mediated translation o the HCV RNA genome.

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Virology of hepatitis C virus.

Simmonds P.

Department of Medical Microbiology, University of Edinburgh Medical Sch
Scotland, United Kingdom.

Hepatitis C virus (HCV) has been identified as the main causative agent of posttransfusion non-A, non-B hepatitis. Through recently developed diagnos assays, routine serologic screening of blood donors has prevented most cases posttransfusion hepatitis. The purpose of this paper is to comprehensively review current information regarding the virology of HCV. Recent findings c the genome organization, its relationship to other viruses, the replication of HCV ribonucleic acid, HCV translation, and HCV polyprotein expression an processing are discussed. Also reviewed are virus assembly and release, the variability of HCV and its classification into genotypes, the geographic distribution of HCV genotypes, and the biologic differences between HCV genotypes. The assays used in HCV genotyping are discussed in terms of reliability and consistency of results, and the molecular epidemiology of HC infection is reviewed. These approaches to HCV epidemiology will prove valuable in documenting the spread of HCV in different risk groups, evaluati alternative (nonparenteral) routes of transmission, and in understanding more about the origins and evolution of HCV.

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FULL-TEXT ARTICLE

Hepatitis C virus core protein: carboxy-terminal boundaries of two processed species suggest cleavage by a signal peptide peptidase.

Hussy P, Langen H, Mous J, Jacobsen H.

F. Hoffmann-La Roche Ltd, Basel, Switzerland.

The expression and processing of hepatitis C virus core protein was analyzed Two protein bands, 21 kDa (P21), corresponding to the full-length core, and kDa (P19), were detected as major products when core protein was expressec the standard rabbit reticulocyte lysate system or in Sf9 insect cells. Core proteins with amino-terminal hexa-histidine tags were expressed which allow the purification of the hexa-histidine P19 core with NI(2+)-NTA columns. W the help of mass spectrometry, the molecular weight of hexa-histidine-P19 w analyzed and its carboxy-terminus could be calculated. Fusion proteins of truncated core/core-E1 species fused to mouse dihydrofolate reductase (mDHFR) showed cleavage in the expected region. Cleavage sites could be determined by amino-terminal protein sequencing of the DHFR-fusion partn Our data show that there are not one but two core products with an apparent molecular weight of about 19 kDa, ending either at amino acid leucine 179 o leucine 182, respectively. These cleavages in the hydrophobic, carboxy-terminal region of HCV core suggest processing by (a) recently proposed eucaryotic signal peptide peptidase(s) (F. Lyko et al. (1995) J. Biol. Chem. 2 19873-19878). Furthermore, our results demonstrate that cleavage at these si and the formation of the P19 species does not require previous processing at signalase site (position 191/192) of the HCV-polyprotein.

PMID: 8862403 [PubMed - indexed for MEDLINE]

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Differential cytotoxic T-lymphocyte responsiveness to the hepati B and C viruses in chronically infected patients.

Rehermann B, Chang KM, McHutchinson J, Kokka R, Houghton M, Ri CM, Chisari FV.

Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, California 92037, USA.

Cytotoxic T lymphocytes (CTL) are thought to control hepatitis B virus (HB infection, since they are readily detectable in patients who clear the virus whereas they are hard to detect during chronic HBV infection. In chronic hepatitis C virus (HCV) infection, however, the virus persists in the face of a CTL response. Indeed, most infected patients respond to one or more HCV-1 (genotype 1a)-derived CTL epitopes in the core, NS3, and NS4 proteins, and the CTL response is equally strong in patients infected by different HCV genotypes, suggesting broad cross-reactivity. To examine the effect of the HCV-specific CTL response in patients with chronic hepatitis C on viral loac and disease activity, we quantitated the strength of the multispecific CTL response against 10 independent epitopes within the HCV polyprotein. We could not detect a linear correlation between the CTL response and viral load disease activity in these patients. However, the CTL response was stronger ir the subgroup of patients whose HCV RNA was below the detection threshol the HCV branched- chain DNA assay than in branched-chain-DNA-positive patients. These results suggest that the HCV-specific CTL response may be a to control viral load to some extent in chronically infected patients, and they indicate that prospective studies in acutely infected patients who successfull clear HCV should be performed to more precisely define the relationship between CTL responsiveness, viral clearance, and disease severity in this infection.

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The influence of AUG codons in the hepatitis C virus 5' nontranslated region on translation and mapping of the translation initiation window.

Rijnbrand RC, Abbink TE, Haasnoot PC, Spaan WJ, Bredenbeek PJ.

Department of Virology, Leiden University, The Netherlands.

The initiation of translation of hepatitis C virus (HCV) is cap-independent an mediated by an internal ribosome entry site (IRES) that is located in the 5' nontranslated region (5' NTR) of the viral genome. This 5' NTR is relatively long and folds into a complex structure involving multiple hairpins and a pseudoknot. Within the sequence encompassing the IRES there are several AUG triplets. Some of these AUG codons are conserved between HCV genotypes and the related pestiviruses. In this study the 5 AUG codons (positions 13, 32, 85, 96, and 215) that are present in the 5' NTR of the HCV strain have been mutagenized to determine their influence on HCV cap-independent translation. The effect of these mutations on the expression of a chloramphenicol acetyl transferase (CAT) gene was tested in vaccinia virus. vTF7-3 infected Hep2 cells transfected with plasmids for the expression of a monocistronic HCV 5' NTR-CAT mRNA. Mutating the AUG codons at positions 13, 32, and 215 does not have a significant effect on CAT expressic inactivating the AUG codons at either position 85 or position 96 severely impaired IRES function. To determine whether ribosomes scan the RNA to select the initiation site, AUG codons were inserted up- and downstream of tl authentic HCV polyprotein translation initiation codon (position 342). Analy of these mutants has revealed that the ribosome is unable to use an AUG cod that is placed either 7 nucleotides upstream or 8 nucleotides downstream of tl inactivated AUG at position 342. These results indicate that when scanning i: involved in the recognition of the translation initiating AUG, it is limited to a narrow region between nucleotides 335 and 350.

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1: J Virol. 1997 Apr;71(4):2838-43. Related Articles, L

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Nonstructural protein 3 of hepatitis C virus blocks the distributi of the free catalytic subunit of cyclic AMP-dependent protein kinase.

Borowski P, Oehlmann K, Heiland M, Laufs R.

Institut fur Medizinische Mikrobiologie und Immunologie, Universitatskrankenhaus Eppendorf, Hamburg, Germany.

Chronic hepatitis resulting from hepatitis C virus (HCV) infection develops i cirrhosis in at least half of infected patients and increases the risk of hepatocellular carcinoma. The pathogenic effects of a number of viruses resu from the disturbance of intracellular signal cascades caused by viral antigens Therefore, we investigated the interaction of nonstructural protein 3 (NS3) o1 HCV with the cyclic AMP-dependent signal pathway. We found a similarity between the HCV sequence Arg-Arg-Gly-Arg-Thr-Gly-Arg-Gly-Arg-Arg-G Ile-Tyr-Arg localized in NS3 and the general consensus sequence of protein kinase A (PKA). Consequently, the catalytic (C) subunit of PKA bound to a bacterially expressed fragment of HCV polyprotein containing amino acid residues 1189 to 1525. When this fragment was introduced into cells, it inhibited the translocation of the C subunit into the nucleus after stimulation with forskolin. The result of this inhibition was significantly reduced histone phosphorylation. Therefore, the presence of NS3 in the cytoplasm of infectec cells may affect a wide range of PKA functions and contribute to the pathogenesis of the diseases caused by HCV.

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1: Protein Eng. 1997 May;10(5):607-14.

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Affinity selection of a camelized V(H) domain antibody inhibitoı of hepatitis C virus NS3 protease.

Martin F, Volpari C, Steinkuhler C, Dimasi N, Brunetti M, Biasiol G, Altamura S, Cortese R, De Francesco R, Sollazzo M.

Istituto di Ricerche di Biologia Molecolare (IRBM) P. Angeletti, Pomezia (Rome), Italy.

The HCV genome encodes, within the NS3 gene, a serine protease whose activity specifically cleaves the viral polyprotein precursor. Proteolytic processing of HCV polyprotein precursor by the viral NS3 proteinase is essential for virion maturation and designing specific inhibitors of this protea as possible anti-viral agents is a desirable and practical objective. With a vie to studying both the function of HCV NS3 protease and to designing inhibito of this enzyme, we directed our interest towards engineering macromoleculaı inhibitors of the viral protease catalytic activity. We describe here the affinit selection and biochemical characterization of one inhibitor, cV(H)E2, a 'camelized' variable domain antibody fragment, isolated from a phage displa synthetic repertoire, which is a potent and selective inhibitor of proteolysis b the NS3 enzyme. In addition to being useful as a biological probe to study th function of HCV protease, this inhibitor can serve as a potential pharmacoph model to design antivirals. Moreover, the results suggest a way of engineerin improved human-derived small recognition units tailored for enzyme inhibiti

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1: Hepatology. 1997 Dec;26(6):1616-20.

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WILEY InterScience

Nucleotide sequence variations in the internal ribosome entry si of hepatitis C virus-1b: no association with efficacy of interferon therapy or serum HCV-RNA levels.

Yamamoto C, Enomoto N, Kurosaki M, Yu SH, Tazawa J, Izumi N, Marumo F, Sato C.

Second Department of Internal Medicine, Faculty of Medicine, Tokyo Medic and Dental University, Japan.

The extreme 5'-proximal sequences of the hepatitis C virus (HCV) genome including the 5'untranslated region (5'UTR) and the first 30 nucleotides of th core region are highly conserved, and serve as an internal ribosome entry site (IRES) that initiates the cap-independent translation of HCV polyprotein. Mutations in the IRES sequence have been shown to cause changes in the efficiency of protein translation in vitro. However, the significance of genetic variations in the IRES is not fully known in clinical settings. Pretreatment se of 25 patients with HCV-1b infection who were treated with interferon were amplified by polymerase chain reaction (PCR), and the IRES sequence was directly sequenced. Correlation of interferon responses or other clinical featu with IRES sequence variability was studied. Eleven of 25 patients were sustained responders (SR) of interferon treatment (negative serum HCV RN/ and normal alanine transaminase levels for 6 months after the end of interfer treatment), and the other 14 patients were nonresponders ([NR], defined as a patient with positive serum HCV RNA within 6 months after the end of interferon therapy). In each patient, one to four nucleotide substitutions were found compared with the consensus sequence of HCV-1b genotype. There w no differences in the number of nucleotide substitutions between either SR a NR (mean, 1.8 in SR, 2.1 in NR; $P = .30$), and no specific variations associat with SR or NR were observed. Although NR had significantly higher serum levels of pretreatment HCV RNA than SR (median, 16 vs. <0.5 Meq/mL; $P = .02$), there was no correlation between the HCV-RNA level and the numbe nucleotide substitutions in the IRES (mean, 1.9 nucleotide substitutions in 12 patients with HCV RNA <0.5 Meq/ mL vs. 2.1 nucleotide substitutions in 13 patients with HCV RNA >0.5 Meq/mL; $P = .61$). Sequence variability of the IRES has no influence on interferon efficacy or serum HCV-RNA concentrations in patients with chronic HCV-1b infection.

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1: J Med Virol. 1998 Jun;55(2):129-33.

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Lack of anti-GOR antibody among subjects with GB virus C/hepatitis G virus RNA.

Nakano T, Mizokami M, Cao K, Noguchi S, Sata M, Park YM, Kim BS, Oyunsuren T, Pereira LB, Ruzibakiev R, Gurtsevitch V, Hayami M.

Second Department of Medicine, Nagoya City University Medical School, Mizuho, Nagoya, Japan.

Homologies were sought between the putative amino acid sequences of GB virus C/hepatitis G virus (GBV-C/HGV) and the GOR epitope or the liver/kidney microsome-1 (LKM-1) epitope, which share partial sequence identity with the hepatitis C virus (HCV) polyprotein. Anti-GOR antibody (a GOR) was assayed among 100 subjects with GBV-C/HGV RNA. Twenty-on and 25 subjects were coinfected with hepatitis B virus (HBV) or HCV, respectively. Homologies were found between the NS5 or E2 polyproteins ol GBV-C/HGV and the GOR epitope or the LKM-1 epitope, respectively. The segments of GBV-C/HGV polyproteins sharing identity with the GOR or the LKM-1 epitope were well conserved among three genotypes of GBV-C/HG\ However, only 1 of 55 subjects (1.8%) with GBV-C/HGV RNA, but not witl HBV or HCV, was positive for anti-GOR. The positivity for anti-GOR amon the group with GBV-C/HGV RNA alone was significantly lower than that among the groups with HCV RNA ($P < 0.01$ and $P < 0.05$, respectively). Onl of 55 subjects (3.6%) with GBV-C/HGV RNA alone exhibited elevation of alanine aminotransferase. The incidence of liver dysfunction among the grou with GBV-C/HGV RNA alone was significantly lower than the incidence among the groups with GBV-C/HGV RNA and hepatitis B surface antigen (HBsAg) or HCV RNA ($P< 0.01$ and $P< 0.01$, respectively). These data indic that 1) there is no association between GBV-C/HGV infection and the preser of anti-GOR, and 2) GBV-C/HGV infection is not related to chronic liver dysfunction.

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JOURNAL OF VIROLOGY, Apr. 1997, p. 2881–2886 Vol. 71, No. 4
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The NS3 Proteinase Domain of Hepatitis C Virus Is a Zinc-Containing Enzyme

MARIUSZ STEMPNIAK, ZUZANA HOSTOMSKA, BEVERLY R. NODES, AND ZDENEK HOSTOMSKY*
Agouron Pharmaceuticals, Inc., San Diego, California 92121

Received 1 October 1996/Accepted 10 January 1997

NS3 proteinase of hepatitis C virus (HCV), contained within the N-terminal domain of the NS3 protein, is a chymotrypsin-like serine proteinase responsible for processing of the nonstructural region of the HCV polyprotein. In this study, we examined the sensitivity of the NS3 proteinase to divalent metal ions, which is unusual behavior for this proteinase class. By using a cell-free coupled transcription-translation system, we found that HCV polyprotein processing can be activated by Zn^{2+} (and, to a lesser degree, by Cd^{2+}, Pb^{2+}, and Co^{2+}) and inhibited by Cu^{2+} and Hg^{2+} ions. Elemental analysis of the purified NS3 proteinase domain revealed the presence of zinc in an equimolar ratio. The zinc content was unchanged in a mutated NS3 proteinase in which active-site residues His-57 and Ser-139 were replaced with Ala, suggesting that the zinc atom is not directly involved in catalysis but rather may have a structural role. Based on data from site-directed mutagenesis combined with zinc content determination, we propose that Cys-97, Cys-99, Cys-145, and His-149 coordinate the structural zinc in the HCV NS3 proteinase. A similar metal binding motif is found in 2A proteinases of enteroviruses and rhinoviruses, suggesting that these 2A proteinases and HCV NS3 proteinase are structurally related.

Hepatitis C virus (HCV) was identified as a major causative agent of posttransfusion and community-acquired non-A, non-B hepatitis throughout the world (see reference 17 for a review). HCV is an enveloped virus with a positive-stranded RNA genome of 9.4 kb which contains a single, large open reading frame (ORF) encoding a precursor polyprotein of about 3,010 amino acids. Based on comparison of deduced amino acid sequences and the extensive similarity in the 5′ untranslated region, HCV has been classified as a separate genus of the family *Flaviviridae*, distantly related to flaviviruses and pestiviruses (7, 26). As was determined by transient expression of cloned HCV cDNAs (11, 15), the precursor polyprotein is cotranslationally and posttranslationally processed into at least 10 viral structural and nonstructural proteins by the action of a host signal peptidase and by two distinct viral proteinase activities (Fig. 1). A novel Zn^{2+}-dependent activity (NS2-3 proteinase) appears to mediate autocatalytic cleavage at the NS2/3 site (10, 16). NS3 proteinase, located in the N-terminal one-third of the 70-kDa NS3 protein (the remaining two-thirds of NS3 encompasses a helicase domain) catalyzes cleavage at four downstream sites in the nonstructural region (5, 11, 15, 35).

The His-57, Asp-81, and Ser-139 residues of the NS3 proteinase are conserved among all sequenced HCV strains and have been proposed to constitute the characteristic serine proteinase catalytic triad, as in the NS3 protein of flaviviruses and pestiviruses (3, 8). That these three residues are essential for HCV NS3 proteinase activity was confirmed by site-directed mutagenesis (16). Based on inhibition studies using series of class-specific protease inhibitors, the NS3 proteinase has been classified as a chymotrypsin-like serine proteinase (12).

Recently, it was shown that NS3 proteinase requires another virus-encoded protein, NS4A, to cleave efficiently at the NS3/4A, NS4A/4B, NS4B/5A, and NS5A/5B junctions (1, 6, 20, 34). In addition to this requirement for a protein cofactor, there were reports of NS3 proteinase sensitivity to divalent metal ions, behavior that is not expected for a chymotrypsin-like serine protease. These sometimes contradictory reports mention, e.g., mild activation by Zn^{2+} and inhibition by Cu^{2+} (13); a requirement for Mg^{2+} (4); inhibition by Zn^{2+}, Ni^{2+} and several chelators, such as EDTA and 1,10-phenantroline (27); and mild inhibition by EDTA (21). In this study, we explored the metal sensitivity of the NS3 proteinase activity in more detail. By using a cell-free transcription-translation system and several forms of purified recombinant protein, we have established that the NS3 proteinase domain of HCV contains a zinc atom which appears to have a structural role.

MATERIALS AND METHODS

Expression constructs. ORFs encoding the NS3-4A-4B, NS4A-4B, and NS3 proteins were amplified by PCR from the plasmid template pBRTM/HCV1-3011 (11), which contains the entire HCV H strain ORF. A methionine codon present in the *Nde*I site was designed into the PCR primers to immediately precede the first codon of each ORF. The *Nde*I-*Eco*RI fragments were inserted into multicopy plasmid pGZ (25). A gene encoding the NS3 protease domain (amino acids 1 to 181) of the HCV J strain designed for expression in *Escherichia coli* was assembled from synthetic oligonucleotides in the pGZ vector. The nucleotide sequence of the gene was modified to reflect the codon usage for *E. coli* and to introduce several unique restriction sites (19a). Standard techniques were used for recombinant DNA manipulations. Splice overlap extension PCR (39) was used to introduce defined mutations into the nucleotide sequence. Each mutation was verified by DNA sequencing.

Cell-free transcription and translation. Cell-free transcription and translation of the HCV H sequences were performed in the TnT T7 Coupled Reticulocyte Lysate System (Promega) by using circular plasmid DNA templates in accordance with the manufacturer's instructions. No viral sequences, such as the 5′ nontranslated region of encephalomyocarditis virus, were present on the plasmid templates; rather, a consensus bacterial ribosomal binding site of the pGZ vector was used to direct translation in this system. The translation products were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and the ^{35}S-labeled proteins were visualized by using a PhosphorImager (Molecular Dynamics) with ImageQuant software.

Protein preparation. Protein expression, purification, and enzymatic characterization will be described in detail elsewhere. Briefly, a pGZ plasmid construct encoding amino acids 1 to 181 of the HCV J strain NS3 protein was expressed in *E. coli* BL 21 (DE3) grown in a complex medium (2xYT) at 28°C in a 30-liter fermentor. After the presence of zinc was established in the first NS3 proteinase preparations, the 2xYT medium was routinely supplemented with 100 μM zinc acetate in the subsequent fermentation runs. A soluble cytoplasmic portion of

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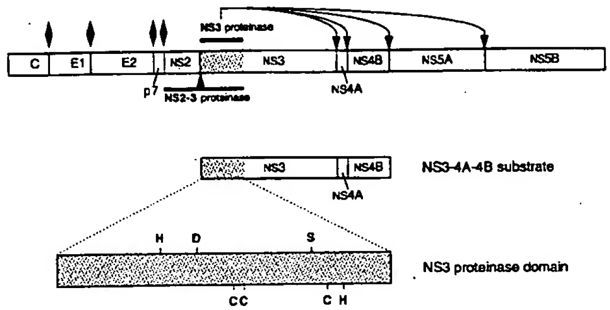

FIG. 1. HCV polyprotein, NS3-4A-4B polyprotein substrate, and NS3 proteinase domain. The full-length precursor polyprotein encoded by the HCV ORF is schematically depicted at the top, and the names of 10 cleavage products are included. Cleavage sites of a host signal peptidase are indicated by solid diamonds. The extent of the NS2-3 proteinase region is shown below the polyprotein; a single NS2-3 proteinase cleavage site is indicated by a short arrow. The NS3 proteinase region is shown above the polyprotein, and NS3 proteinase cleavage sites are indicated by curved arrows. The NS3 proteinase domain is shaded. Residues (in the single-letter amino acid code) of the presumed serine proteinase catalytic triad (His-57, Asp-81, and Ser-139) are above the bar, and residues of the putative zinc binding site proposed in this report (Cys-97, Cys-99, Cys-145, and His-149) are below the bar. The residue numbers are in the NS3 proteinase numbering, which can be converted to the HCV J strain polyprotein numbering by adding 1,026.

the induced *E. coli* cell paste was subjected to chromatography on Fast Flow SP Sepharose, FPLC Mono S, and Sepharose S-200. The purified protein was stored in 50 mM sodium acetate buffer (pH 6.0)–10 mM dithiothreitol–350 mM sodium chloride at −70°C until used for analysis. The protein concentration was determined with Pierce Coomassie Assay reagent by using serum albumin as the standard. Modification of the HCV NS3 proteinase with $HgCl_2$ was carried out in 20 mM morpholineethanesulfonic acid (MES) buffer, pH 6.0, for 30 min at 4°C in the presence of a twofold excess of $HgCl_2$ over protein. The unreacted $HgCl_2$ was removed on a Pierce Desalting Column in 20 mM MES, pH 6.0. For metal analysis, 0.4 to 1 mg of pure protein was treated with 5 mg of Chelex 100 resin (Bio-Rad), a divalent metal chelating resin, by mixing in suspension for 1 h at 4°C. Following centrifugation, the supernatant was lyophilized to dryness.

Determination of metal content. Analysis of metal content in HCV NS3 proteinase samples was performed by Elemental Research, Inc., North Vancouver, British Columbia, Canada. The protein sample was subjected to metal analysis by inductive coupled mass spectroscopy (ICPMS). The metal content was reported in nanograms per milliliter of sample. By using the calculated molecular mass of the protein (based on the primary amino acid sequence), the mass of the metal, and the protein concentration, the moles of metal per mole of protein were calculated.

RESULTS

Effect of divalent metal ions on autoprocessing of HCV polyprotein. HCV polyprotein fragment NS3-4A-4B (Fig. 1) was used as model substrate to explore the extent of autoprocessing by the NS3 proteinase activity. This substrate contains the NS3-NS4A cleavage site, believed to be processed in *cis*, and the NS4A-4B site processed in *trans* (2). The polyprotein was synthesized in a coupled transcription-translation system containing rabbit reticulocyte lysate and phage T7 RNA polymerase, from a plasmid encoding the NS3-4A-4B fragment. Different metal ions were added to the reaction mixture, and their effects on processing were monitored by separation of products by SDS-PAGE. Of the 12 divalent metal ions tested at a 100 μM final concentration as the respective chloride salts, Zn^{2+} was the most efficient at stimulating the basal level of polyprotein processing. Less efficient but still detectable stimulation was observed with Cd^{2+}, Pb^{2+}, and Co^{2+} (Fig. 2). Monitoring of the effect of increasing concentrations of Zn^{2+} on polyprotein processing showed a 50% stimulatory concentration of ~20 μM (Fig. 3). Expression of the NS3-4A-4B polyprotein in the absence of Zn^{2+}, followed by Zn^{2+} addition and further incubation for up to 4 h, showed no activation of polyprotein processing (data not shown). This suggests that Zn^{2+} needs to be present during protein folding to activate

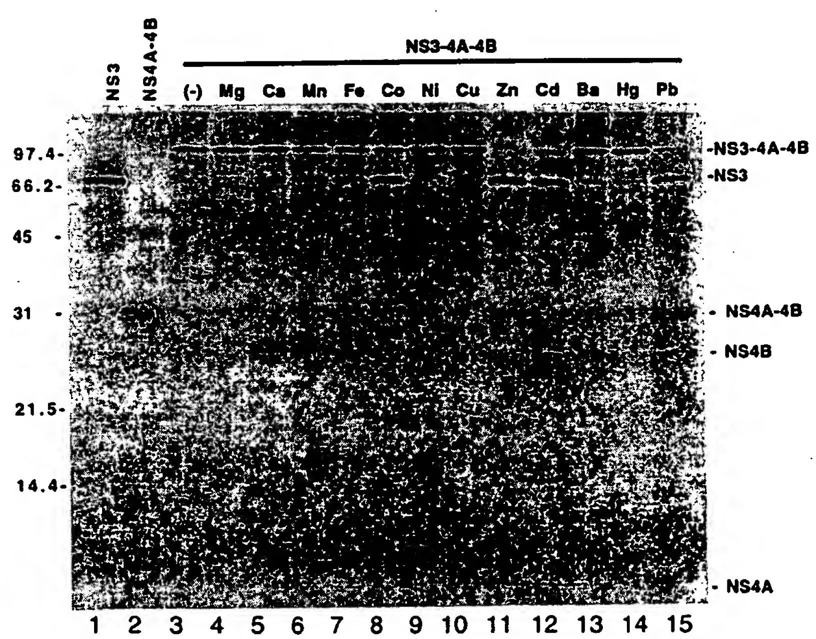

FIG. 2. Effects of divalent metal ions on autoprocessing of the NS3-4A-4B polyprotein substrate. Plasmids directing expression of the NS3 protein (lane 1) and the NS4A-4B protein (lane 2) in the coupled transcription-translation reactions were used as controls to indicate positions of an expected product and intermediate of NS3-4A-4B autoprocessing. The reactions expressing the NS3-4A-4B substrate were performed in the presence of various metal chlorides (indicated above) at a 100 μM final concentration. After 3 h of incubation at 30°C, the ^{35}S-labeled translation products were separated by SDS–12% PAGE. Molecular weights (in thousands) are shown on the left, and positions of the substrate, processing intermediate, and products are shown on the right.

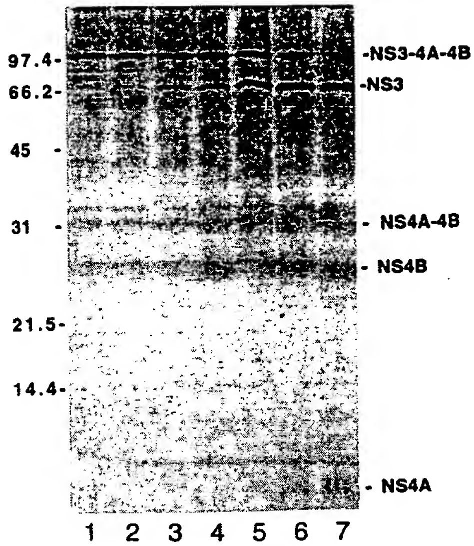

FIG. 3. Effect of increasing concentrations of $ZnCl_2$ on the extent of NS3-4A-4B substrate autoprocessing. The final Zn^{2+} concentrations in the reaction mixtures are shown at the top. The other experimental conditions for the coupled transcription-translation reactions and the lane designations are the same as in Fig. 2.

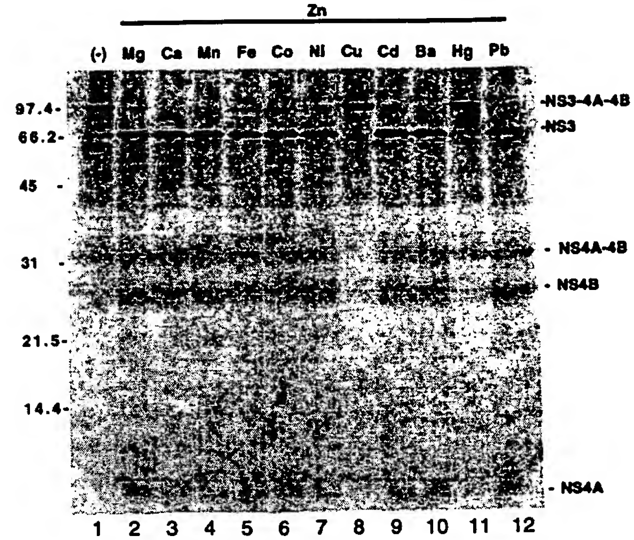

FIG. 4. Effects of divalent metal ions on Zn^{2+}-activated autoprocessing of the NS3-4A-4B substrate. Plasmid-directed expression of the NS3-4A-4B substrate was performed in the presence of 100 μM $ZnCl_2$ and different metal chlorides at 100 μM as indicated at the top. Lane 1 contained a control reaction to which no metals were added. The other experimental conditions for the coupled transcription-translation reaction and the lane designations are the same as in Fig. 2.

NS3 proteinase and has no effect when added posttranslationally. Elemental analysis of the transcription-translation reaction mixture before metal addition indicated that there is approximately a 40 μM concentration of total zinc. However, this analysis cannot distinguish how much of that zinc is in a free form and how much is protein bound in components of the rabbit reticulocyte lysate. The concentration of available Zn^{2+} in the reaction mixture is apparently not sufficient for complete activation of the NS3 proteinase but may explain the basal level of polyprotein processing (e.g., Fig. 2, lane 3).

Inhibition of NS3-4A-4B polyprotein processing by Cu^{2+} and Hg^{2+} was clearly visible in the reaction mixture supplemented with 100 μM Zn^{2+} (Fig. 4). Further characterization of this effect in experiments with various concentrations of inhibitory metals showed the approximate 50% inhibitory concentrations in this system to be ~7 μM for Cu^{2+} and ~20 μM for Hg^{2+} (data not shown). These results confirm and extend previous observations that HCV polyprotein processing by NS3 proteinase can be influenced by divalent metal ions and suggest that Zn^{2+} is an activator, while Cu^{2+} and Hg^{2+} are potent inhibitors, of this enzyme.

Elemental analysis of the purified NS3 proteinase domain. To explore the possibility that NS3 proteinase contains a metal cofactor, the purified recombinant NS3 proteinase domain was subjected to ICPMS and analyzed for the presence of 70 elements. Of the metals, only zinc had a significant presence in the protein samples. There were only traces of calcium and iron (0.17 and 0.04 mol/mol of protein, respectively) and no significant presence of Cd, Pb, Co, Mg, Mn, Cu, or any other metal. The zinc atom was apparently taken up in the course of NS3 proteinase synthesis and folding in the bacteria, as no additional zinc ions were added to the sample during or after protein purification. As seen in Table 1, the wild-type form of the NS3 proteinase domain contains about 1.05 mol of zinc per mol of protein. The active-site double mutant, which has two presumed active-site residues, Ser-139 and His-57, changed to alanine (H57A, S139A) contains about 1 mol of zinc per mol of protein, suggesting that zinc ion is not bound at the active site. The results of this analysis, as summarized in Table 1, indicate that the NS3 proteinase domain contains one zinc atom per protein molecule.

Zinc binding site. The sulfhydryl group of cysteine and the imidazolyl group of histidine are the most common ligands in structural zinc binding sites, whereas acidic side chains are more frequent ligands at catalytic zinc sites (36). In our search for possible zinc binding residues in the NS3 proteinase sequence, we therefore focused on cysteine and histidine residues. There are seven cysteines in the NS3 proteinase domain of the HCV J, BK, and H strains, four of which are conserved in all known HCV sequences. Mutation analysis by Hijikata et al. (16) suggested that, in contrast to Cys-16, Cys-47, Cys-52, and Cys-159, which appear to be dispensable, Cys-97, Cys-99, and Cys-145 are critical for efficient polyprotein processing. The latter three residues thus became prime candidates for involvement in zinc coordination. The zinc contents of the NS3 proteases with mutations in dispensable cysteines (C159S and

TABLE 1. Zinc content[a] in the wild type and several mutated forms of the purified NS3 proteinase domain of HCV

Mutation(s)[b]	Zn/enzyme molar ratio
None (wild type)	1.05[c]
H57A, S139A	1.02
C159S	0.86
C16A, C47S, C52L, C159S	1.04
C16A, C47S, C52L, C159S/$HgCl_2$	0.16[d]
H149A	ND[e]

[a] Zinc content was determined as described in Materials and Methods.
[b] The single-letter amino acid code is used to describe mutations introduced into the NS3 proteinase domain.
[c] Average of 14 measurements for different protein preparations.
[d] Zinc content determined in the quadruple mutant covalently modified with $HgCl_2$.
[e] ND, not detectable; see text for details.

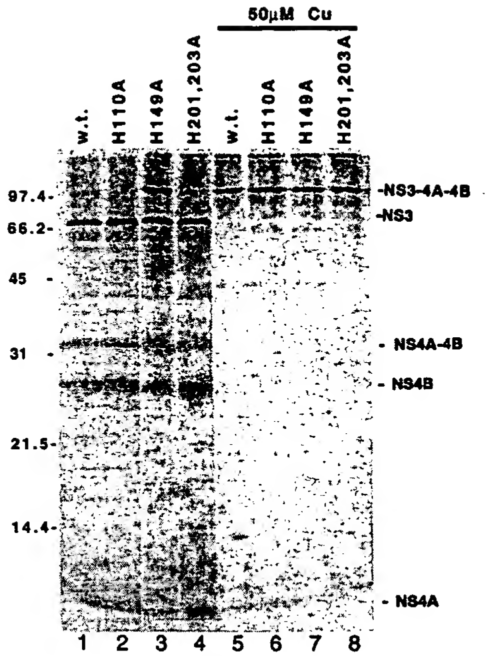

FIG. 5. Effect of His substitutions in the N-terminal region of NS3 on autoprocessing of the NS3-4A-4B substrate. His-to-Ala substitutions were introduced into the plasmid directing expression of the NS3-4A-4B substrate at the positions indicated at the top. $ZnCl_2$ (100 µM) was present in all of the reaction mixtures, and 50 µM $CuCl_2$ was added to the reaction mixtures in lanes 5 to 8. The other experimental conditions for the coupled transcription-translation reaction and the lane designations are the same as in Fig. 2. w.t., wild type.

		97 99		139 145 149
HCV-J	(NS3)	SMTPCTCGSSD	.....(35 aa).....	SGGPLLCPSGHV
HGV	(NS3)	SLTPCTCQAES	.....(34 aa).....	SGSPVLCDEGHA
GBV-A	(NS3)	CLQACKCQPTG	.....(33 aa).....	SGSPILCDEGHA
GBV-B	(NS3)	SLTRCSCGETK	.....(35 aa).....	SGAPILCSSGHV
HRV-2	(2A)	YIPSCDCTQAT	.....(47 aa).....	CGGKLLC--KHG
PV-1	(2A)	SIARCNCNAGV	.....(47 aa).....	CGGILRC--HHG
		55 57		109 115 117

FIG. 6. Alignment of selected NS3 and 2A proteinases. A representative amino acid (aa) sequence was chosen for each genus of the *Flaviviridae* and *Picornaviridae* families that contains the presumed zinc binding motif in the respective NS3 and 2A proteinases. The GenBank accession or reference numbers of the sequences shown are as follows: HCV-J, D90208; hepatitis C virus strain J, reference 18; HGV, U44402; hepatitis G virus, reference 22; GBV-A, U22303; GBV-B, U22304; GB viruses A and B, reference 31; HRV-2, X02316; human rhinovirus type 2, reference 32; PV-1, P03399; poliovirus type 1, reference 19. Residues presumably involved in zinc coordination are in boldface type. The active-site nucleophiles (Ser for NS3 proteinases and Cys for 2A proteinases) are italicized and underlined. Residues are numbered according to the amino acid sequence of the HCV-J NS3 proteinase (top), or the 2A proteinase of the Mahoney strain of type 1 poliovirus (bottom). The HRV-2 and PV-1 sequences were taken from the alignment of 2A proteinases of rhinoviruses and enteroviruses (38).

the C16A, C47S, C52L, C159S quadruple mutant) were equivalent to that of the wild type (Table 1), suggesting that none of these four residues participates in zinc binding.

Because we expected four-coordinate tetrahedral geometry around the zinc ion and only three candidate cysteine ligands were identified, we considered a histidine residue as a possible fourth ligand. The sequence of the core domain of the NS3 proteinase (residues 1 to 181) contains three histidine residues, His-57, His-110, and His-149. Of these, His-57 was confirmed by mutational analysis to be essential for NS3 proteinase activity (9, 16), consistent with its being a member of the serine proteinase catalytic triad. As described above, the wild-type zinc content of the purified H57A, S139A double mutant form of the NS3 proteinase domain (Table 1) argues against involvement of His-57 in zinc coordination and against a catalytic role for the zinc ion. To explore the roles of the remaining His residues (His-110 and His-149) in polyprotein processing that would suggest zinc binding, His-to-Ala substitutions were introduced into the NS3-4A-4B polyprotein substrate. In addition, His-201 and His-203, which lie outside the amino acid sequence of the minimal NS3 proteinase, were also included in this experiment because of similarity to a possible HXH metal binding motif and their immediate proximity to the NS3 proteinase core domain. As is apparent from Fig. 5, the H110A mutant and the H201A, H203A double mutant show the wild-type level of polyprotein processing, while the processing of the substrate carrying the H149A mutation is reduced. The possibility that His-149 has a role in zinc binding was further explored by analysis of the purified recombinant protein. Expression in *E. coli* of the NS3 proteinase domain carrying the H149A mutation resulted in accumulation of practically all protein in the insoluble fraction. No zinc was detected in this material after it was solubilized and subjected to ICPMS metal analysis (Table 1), suggesting that absence of zinc correlates with improper protein folding. Interestingly, a small amount, representing less than 1% of the total expressed NS3 H149A proteinase, could be recovered from the soluble fraction of the bacterial lysate and, based on preliminary analysis, this soluble protein has a wild-type zinc content. Impaired protein folding can thus explain the incomplete processing of the NS3-4A-4B polyprotein substrate carrying the H149A mutation observed in the reticulocyte lysate. This phenotype is consistent with the possibility that His-149 is a fourth residue of the proposed Zn binding site, although its contribution to the metal coordination appears to be less important than that of any of the three cysteine residues (Cys-97, Cys-99, and Cys-145) (16).

The published data on mutagenesis of Cys residues and our histidine mutagenesis data, combined with zinc content determination in selected mutated forms of the purified domain, lead us to propose that Cys-97, Cys-99, Cys-145, and His-149 constitute a structurally important zinc binding site in the HCV NS3 proteinase.

Sequence comparison of HCV NS3 proteinase with proteinases of other RNA viruses. Mutational analysis of Cys and His residues in the poliovirus 2A proteinase revealed that in addition to His-20 and Cys-109, presumed members of the catalytic triad, there are four other Cys and His residues (Cys-55, Cys-57, Cys-115, and His-117) whose alteration eliminates enzymatic activity (38). The four latter residues form a CXC-...CXH motif (X = any amino acid) that is conserved among 2A proteinases of known enteroviruses and rhinoviruses and was suggested to maintain the active conformation of the 2A proteinase structure and implies the binding of a metal ion, such as Zn^{2+} (38). Later, it was demonstrated that the purified rhinovirus 2A proteinase, indeed, contains a zinc atom that is required for the correct folding and stability of an active enzyme (33, 37), although binding of the zinc atom by the CXC...CXH motif was not directly demonstrated.

As seen in Fig. 6, the motif CXC...CXXXH, which we propose to be the HCV NS3 proteinase zinc binding site, is quite similar to the proposed zinc binding motif in enteroviruses and rhinoviruses. The first half of the motif, CXC, is commonly found in multiple repeats in metallothioneins, a class of proteins that contain several tetrahedrally bound Zn and Cd ions (30). The Cys residue in the second half of the

motif has the same position with respect to the presumed active-site nucleophile (Ser or Cys) in all of the aligned sequences, suggesting the same three-dimensional arrangement and possibly a common evolutionary origin of the NS3 and 2A proteinases.

The similarity of the conserved CXC...CXH motif in the zinc containing rhinovirus 2A proteinase to the CXC...C XXXH motif in the HCV NS3 proteinase strengthens our suggestion that Cys-97, Cys-99, Cys-145, and His-149 represent a zinc binding site in HCV NS3 proteinase. The same CXC ...CXXXH motif can also be found in the recently identified GB viruses GBV-A and GBV-B (31) and the GBV-C/hepatitis G virus (22) (Fig. 6), each of which constitutes a new genus of *Flaviviridae* closely related to HCV (28, 31). Interestingly, no similar Zn binding motif is present in the NS3 proteinase domains of other members of the *Flaviviridae* family, such as in yellow fever virus, dengue virus, and tick-borne encephalitis virus of the classic flaviviruses, nor in bovine viral diarrhea virus and hog cholera virus of animal pestiviruses.

DISCUSSION

In this study, a cell-free coupled transcription-translation system was used to examine the effects of several divalent metal ions on the autocatalytic processing of a polyprotein substrate. The effects ranged from significant activation (by Zn) to strong inhibition (by Cu). Analysis of the metal content of the purified NS3 proteinase domain indicated the presence of a single zinc atom per molecule of enzyme which, in turn, implied the existence of a metal binding site. We propose that this site is formed by Cys-97, Cys-99, Cys-145, and His-149, based on results from site-directed mutagenesis and sequence comparisons with 2A proteinases. The notion that Cys-97, Cys-99, Cys-145, and His-149 constitute a Zn binding site is further supported by a two-beta-barrel trypsin-like homology model of the NS3 proteinase domain (22a) based on the rhinovirus 3C proteinase structure (24). In this model, the Cys-97, Cys-99, Cys-145, and His-149 residues cluster close together but distant from the presumed active site, which is consistent with a structural rather than a catalytic role for the bound zinc.

The existence of a metal binding site, which presumably coordinates a single zinc atom under physiological conditions, may explain most, if not all, of the observed effects of various divalent metal ions in vitro. Depending on their affinities and atomic properties, they may bind at the zinc site in either a productive way, resulting in activation, or in a nonproductive way, resulting in inhibition. In the protein treated with $HgCl_2$, free sulfhydryl groups of Cys residues are covalently modified by mercury, which prevents them from zinc atom coordination (Table 1). The observed inhibition by Hg ions would then be explained by elimination of ligands, resulting in an inability to form a zinc site. Inhibition by Cu^{2+} may have a similar explanation. It may also be that Cu replaces Zn while maintaining coordination of Zn ligands but without optimum geometry.

However, since Cu^{2+} remains the most potent inhibitor of HCV NS3 proteinase reported thus far, its effect deserves further consideration. NS3 proteinase activity is completely inhibited by Cu^{2+} at low micromolar concentrations which, in addition to interference with a structural site, may also invoke direct interaction with the active-site residues. Interestingly, a single mutation was described in trypsin that made this prototype serine protease susceptible to Cu^{2+} inhibition (14). In that study, the Arg-96 to His substitution was introduced into the recombinant rat trypsin, which resulted in the placement of a new imidazole group on the surface of the enzyme near the essential active site, His-57 (coincidentally, the His residue of the presumed catalytic triad of the HCV NS3 proteinase has the same number). The spatial orientation of these two His side chains enables formation of a stable metal-binding site that chelates divalent first-row transition metal ions. The presence of Cu^{2+} at this site prevents the imidazole group of His-57 from participating as a general base in catalysis. The Cu^{2+} inhibition of the R96H trypsin is reversible by EDTA.

It is not clear whether the observed inhibition of the NS3 proteinase by Cu^{2+} acts through a similar mechanism that would involve active site His-57 and some other naturally occurring copper ligand, e.g., another histidine. The complete inhibition of processing by Cu^{2+}, even in the presence of a large molecular excess of Zn^{2+} (data not shown), may suggest participation of active site His-57. However, none of the other His residues present in the NS3 proteinase domain seems to be critically involved in Cu^{2+} binding, as both the H110A and H149A mutants (and also the H201A, H203A double mutant) are still susceptible to inhibition by Cu^{2+} (Fig. 5). Binding of Cu^{2+} away from the catalytic residues, most probably at the Zn binding site, and exertion of its allosteric inhibitory effect by disruption of structural features in the NS3 proteinase normally stabilized by Zn^{2+} thus remains the most likely explanation for the Cu^{2+} inhibition.

Our finding that the NS3 proteinase domain contains a zinc atom should be related to the observation by Hijikata et al. (16) that NS2-3 proteinase, a second virus-encoded activity that is responsible for autocatalytic cleavage at the NS2-NS3 site, is stimulated by $ZnCl_2$ and inhibited by EDTA, a chelator of divalent metal ions. This observation led the investigators to propose that NS2-3 is a novel zinc-dependent metalloproteinase. However, as emphasized also by Reed et al. (29), these results are not sufficient evidence for classification of NS2-3 as a metalloproteinase, since the observed stimulation of proteinase activity by $ZnCl_2$ and inhibition by EDTA could indicate a structural rather than a catalytic role for zinc (similar to our proposal for the role of zinc in the NS3 proteinase domain).

The NS2-3 proteinase was mapped to a region encompassing 129 amino acids of NS2 and the whole NS3 proteinase domain (10, 16). It is not clear whether NS2 has a different zinc binding site or whether the zinc atom present in the NS3 domain is identical to a zinc required for NS2-3 activity. In their mutagenesis study, Hijikata et al. (16) noted that the C97A, C99A, and C145A mutations in the NS3 domain, which are the residues we propose to constitute a zinc binding site, reduce both NS2-3 and NS3 proteinase activities, which is consistent with a single zinc. As NS2-3 is both an enzyme and a substrate in the autocatalytic cleavage at the NS2-3 site, the zinc atom within the NS3 proteinase may have a structural role in stabilizing the NS3 domain so that it can be efficiently recognized as a substrate by NS2-3 activity. On the other hand, the Cys-993 and His-952 (polyprotein numbering) residues present in the NS2 protein were suggested to coordinate a zinc atom important for NS2-3 proteinase activity (16). If confirmed, it would mean that there is a separate zinc binding site in the NS2 protein.

While this report was being prepared, a refined crystal structure of the HCV NS3 proteinase domain became available (23). The structure supports the main conclusions of this report, namely, the presence of a structural zinc atom coordinated by Cys-97, Cys-99, and Cys-145. The fourth ligand is a water molecule which is hydrogen bonded to His-149. Although His-149 appears not to play a direct chelation role in this crystal form, it is positioned to readily coordinate the metal as a substitute for the water ligand. A reduced level of polyprotein processing seen with the H149A mutant (Fig. 5, lane 3), which is less dramatic than the effects of any of the C97A, C99A, and C145A mutations (16), is thus consistent

with the possibility that His-149 is an integral part of zinc coordination only during initial folding.

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